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EFFECT OF MALE ACCESSORY GLAND EXTRACTS ON INDUCTION OF OVIPOSITION IN THE GYPSY MOTH, *LYMANTRIA DISPAR* (LYMANTRIIDAE)

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ABSTRACT. In gypsy moths, *Lymantria dispar*, injection of saline extracts of male reproductive glands only slightly increased early oviposition by virgin females. Freshly mated females, ligated between the thorax and abdomen to prevent transmission of humoral factors, oviposited at the same time (37 to 53 min) as mated, nonligated, control females (59 min). Transplantation of spermathecae from either mated or senescent, actively-ovipositing unmated females into virgin moths did not elicit early oviposition. Females stimulated by insertion of a probe or injection of saline into the bursa copulatrix failed to oviposit. These data enhance previous findings by others suggesting that initiation of oviposition in gypsy moths is mediated neurally.

Additional key words: sex peptide, ligation, transplantation, spermathecaee.

Mechanisms for induction of oviposition differ among the Lepidoptera. In most cases, the presence of eupyrene sperm and/or testicular fluids in the female reproductive tract is needed to initiate oviposition. For *Hyalophora cecropia* (L.) (Saturniidae), the bursa copulatrix appears to secrete a bursa factor after being filled with sperm; oviposition is initiated by an undefined humoral mechanism (Riddiford & Ashenhurst 1973, Sasaki et al. 1983). A bursa factor also has been postulated for *Manduca sexta* (L.) (Sphingidae) (Sasaki & Riddiford 1984). However, in a follow-up study, Stringer et al. (1985) found that bursa transplants from mated females, which were expected to be humorally active, did not induce oviposition in virgins. They suggested other factors, such as juvenile hormone (JH), also may be needed and that the expanded bursa might transmit neural signals to the corpora allata to maintain the titer of JH. In the leek moth, *Acrolepiopsis assectella* (Zeller) (Acrolepiidae), spermatozoa must migrate to the spermatheca to stimulate oviposition (Thibout 1979). Eupyrene sperm and/or testicular fluids must be present (tissue not specified) for oviposition by larch bud moths, *Zeiraphera diniana* (Guenée) (Torticidae) (Benz 1969), and cabbage loopers, *Trichoplusia ni* (Hübner) (Noctuidae) (Karpenko & North 1973).

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There is ample evidence for both neural and humoral control of reproductive events in Lepidoptera as exemplified by studies of pheromone biosynthesis regulation in *Helicoverpa* (Noctuidae) (Teal et al. 1990, Christensen et al. 1991). The investigators suggested that pheromone biosynthesis-activating neuropeptide is released by efferent nerves acting on the terminal abdominal ganglion, which then signals the pheromone gland to synthesize pheromone. They also discovered a bursa factor that suppresses pheromone production through an undefined mechanism.

Males of several insects transfer factors to females during copulation that induce oviposition and inhibit receptivity to additional mating. Such substances have been reported in Diptera, Orthoptera, Lepidoptera, and Coleoptera (Leahy & Craig 1965, Leahy 1967, Nelson et al. 1969, Yamaoka & Hirao 1977, Morrison et al. 1982, reviewed by Gillott 1988). These factors are thought to be secreted by the accessory reproductive glands of the male. In Diptera, they are referred to as sex peptides based on the pioneering work by Fox (1956, Fox et al. 1959). Chen et al. (1988, Chen & Balmer 1989, Schmidt et al. 1993) have conducted the most extensive research on dipteran sex peptides and have been able to identify, characterize, and synthesize the sex peptides in three species of *Drosophila* (Drosophilidae). The sex peptides probably are transmitted through the bursa to the hemolymph and act on the brain to trigger oviposition (Chen 1991, Aigaki et al. 1991).

The physiological basis for induction of oviposition in gypsy moths, *Lymantria dispar* L. (Lymantriidae), is not fully understood. Adults are sexually mature upon eclosion and generally mate on the day of emergence (Leonard 1981). Females begin to lay an egg mass within 4 to 6 h after mating and complete the process within 3 days. If females have not mated within 5 days, they switch from virgin to mated behavior and spew some of their mature eggs before dying (Giebultowicz et al. 1990a). Although Giebultowicz et al. (1990b) suggest that sperm must be present in the spermathecae to initiate oviposition in gypsy moths, no studies specifically have examined whether humoral factors are involved.

Given prior research in J. Miller's laboratory on oviposition-stimulating substances (Spencer et al. 1992), the recent success in sex peptide characterization achieved by Chen et al. (1988, Chen & Balmer 1989), and the lack of comparable studies on the gypsy moth, we addressed the possibility that induction of oviposition in *L. dispar* might be influenced by a humoral factor from the male accessory glands. Discovery of substances promoting premature oviposition of unfertilized eggs might provide clues towards control of this economically important pest (Leonard 1981, Miller et al. 1994).

MATERIALS AND METHODS

Rearing. Gypsy moth egg masses, obtained from the USDA-APHIS facility at the Otis ANGB in Massachusetts, were surface-sterilized by soaking in 10% formalin for 1 h followed by a cold water rinse for 1 h. Insects were maintained at 24±2 C, 55±5% RH, and a L:D 16:8 h cycle. Groups of 10 newly emerged larvae were placed into 60 ml clear plastic cups and reared on a high wheat germ diet (Bell et al. 1981). During the second instar, individual larvae were transferred into cups with sufficient food for growth to pupation. Pupae were sexed according to size (females are approximately 50% larger than male pupae). Female pupae were placed into a group cage and males were placed individually into 150 ml plastic cups until adult eclosion. Since gypsy moths are sexually mature upon eclosion and because virgin females begin senescent oviposition at about 5 days old, experiments were conducted on males and females 0–2 days old, unless noted otherwise.

Ligation. Newly eclosed female moths (less than 18 h old) were ligated 1, 15, 30, 60 and 180 min after the onset of mating to disrupt humoral communication between the abdomen and head. A 5-0 nylon suture was looped around the thoracic-abdominal junction of female moths, then tightened sufficiently to stop hemolymph flow but not enough to sever the ventral nerve cord. In positive (normally mated) and negative (virgin) control moths, ligatures were applied but not tightened. Anesthesia was not used. The ligature was not tightened prior to copulation (at 0 min) because a preliminary experiment suggested that it reduced either a female's willingness to copulate or her attractiveness to the male. We judged it unlikely that any humoral factors would be released into the hemolymph from the bursa and be transported out of the abdomen during the first minute of copulation. All moths were injected in the abdomen with 0.5–1.0 ml India ink (undiluted) at the end of the experiment to identify moths with incomplete ligations, and some were injected immediately after ligation to measure failure rate of ligation. Preliminary tests showed that India ink readily circulated through all three tagmata of nonligated females.

Extract preparation and injection. Male whole reproductive tract, accessory gland/ejaculatory duct complex, vas deferens/seminal vesicle complex, and testis were dissected from freshly frozen moths and placed into a microcentrifuge vial containing Ringer's saline kept on ice. Extracts were prepared by homogenizing the tissues for 20–30 seconds with an ultrasonic probe, setting the tuner 3 and the power 35 (Blackstone Ultrasonics Inc., Sheffield, Pennsylvania), then centrifuging at 6000 g for 10 minutes. The concentration of the supernatant was adjusted so that 7.0 μl of extract corresponded to 1 male equivalent of tis-

sue. Extracts were used immediately or removed and stored for up to 5 days at –20°C before use. Female moths were anesthetized with CO_2 for 2 min, then injected with a 30 gauge needle into the ventrolateral side of the pleuron between abdominal segments A2 and A3.

Surgical procedures. Female moths were anesthetized with diethyl ether for 30–60 sec prior to surgery. To examine whether isolated abdomens would oviposit, female moths were allowed to mate, then their abdomens were resected at 1, 3, 5, and 7 h after the onset of mating. For tagmatal isolation studies, either the thoracic-abdominal junction or the head-thorax junction was tied off with a suture and resected anteriorly. Since the spermatheca is the target organ of sperm following dissociation of the sperm in the bursa copulatrix and oviposition is markedly reduced in females without a spermatheca but not in females without a bursa copulatrix (Giebultowicz et al. 1990b), spermathecae were transplanted to determine whether they were humorally active. One-day-old virgin females received a spermathecal transplant from one of three donor types: 1-day-old mated, ovipositing females; senescent, ovipositing virgins; 1-day-old nonovipositing virgins. For the transplantation study, spermathecae were removed from donor moths through an incision on the ventral side above the bursa opening. These were transplanted immediately into virgins through a scalpel puncture anterior and lateral to the bursa. The sham control surgery consisted of a scalpel puncture followed by forceps insertion. All wounds were sealed with melted beeswax.

Quantification of oviposition. Since oviposition in normally mated females generally begins between 3–5 h postmating, all treatments were administered between 0 and 7 h after initiation of copulation to maximize the probability of causing a behavioral response. In the ligation and isolation experiments, ovipositional activity was monitored continuously on the first day and daily thereafter. In other experiments, females were examined daily to record the onset of oviposition. Egg masses were weighed to an accuracy of 0.1 mg.

RESULTS

Effect of ligation on oviposition. To determine whether the presence of a ligature would adversely affect mating and oviposition, the time ligated females remained *in copulo* was compared to that of normally mated females. There were no significant differences in copulating time among ligated females compared to nonligated females, regardless of when females were ligated (Table 1). Although females ligated at 30 min tended to disengage during the ligation procedure or soon after, there was no apparent effect on other parameters measured. Overall, ligation had little effect on duration of copulation.

TABLE 1. Gypsy moth mating, oviposition and egg weight at 3 days following ligation. Newly eclosed females were ligated at the times indicated after the onset of mating. In positive and negative control moths, ligatures were applied but not tightened. Results presented as means ± standard deviation. Within each column, treatment means with the same letter are not significantly different ($p<0.05$) using Student-Newman-Keuls test (Ott 1988) for multiple comparison of means following analysis by a general linear model. Time ligated refers to time after initiation of copulation.

Treatment	N	Mean copulation time (min)	Elapsed time to oviposition (h)	Egg mass weight (mg)
Time ligated				
1 min	7	51 ± 14 [a]	14 ± 26 [a]	522 ± 275 [a]
15 min	5	53 ± 21 [a]	10 ± 10 [a]	579 ± 88 [a]
30 min	5	37 ± 13 [a]	4 ± 2 [a]	568 ± 235 [a]
60 min	4	46 ± 17 [a]	5 ± 3 [a]	663 ± 100 [a]
180 min	3	53 ± 17 [a]	5 ± 2 [a]	548 ± 40 [a]
Control				
mated, not ligated	9	59 ± 21 [a]	6 ± 3 [a]	712 ± 107 [a]
virgin, not ligated	5	—	72 ± 1 [b]	15 ± 13 [b]
virgin, ligated	7	—	57 ± 26 [b]	101 ± 200 [b]

The effect of ligation on oviposition activity in moths was investigated by measuring elapsed time from initiation of mating to initiation of oviposition and the weight of the egg mass at three days posttreatment. Mated moths in both the ligated and nonligated groups (Table 1) had similar elapsed times to oviposition and similar egg mass weights. For moths ligated at 1 and 15 min, oviposition appeared to be delayed somewhat compared to later ligation times. However, in each case there was one female per group that oviposited much later than the rest. If those data are removed from the analysis, elapsed time to oviposition is 4±3 h and 6±4 h for the 1 min and 15 min ligation, respectively. Virgin moths placed in oviposition cages at the same time as mated moths took significantly longer to begin ovipositing; only half of the females had laid any eggs at the end of three days, which is characteristic of senescent virgins. The results for ligated virgins were more variable than unligated virgins because two moths in the ligated group began ovipositing late in the first day whereas the other five began at around 72 h. Perhaps the ligatures had some effect on inducing early oviposition but not to the extent that mating does. In both virgin groups, mean egg weights were significantly lower as expected (Table 1).

Several moths with incomplete ligations were not included in the above analyses. However, in effect, they served as parallel controls revealing the effect of applying the ligature at each time period without blocking hemolymph flow. Mean latency to oviposition and mean egg

TABLE 2. Oviposition and egg weight of gypsy moths with complete and incomplete ligations. Moths with incomplete ligations are shown next to those with complete ligations to illustrate the effect of ligation without blocking hemolymph exchange. Treatment means with the same letter are not significantly different at $p<0.05$ by SNK (see Table 1). Time ligated refers to time after initiation of copulation. Asterisks indicate samples of n=1.

	Hours to oviposit		Egg weight (mg)	
Treatment	Complete ligation	Incomplete ligation	Complete ligation	Incomplete ligation
Time ligated				
1 min	14	7	523 [a]	712 [a]
15 min	10	6	579 [a]	667 [a]
30 min	4	7	568 [a]	698 [a]
60 min	5	2*	663 [a]	698 [a]
180 min	5	7	548 [a]	595 [a]
virgin, ligated	57	49*	101 [b]	24 [b]

weights were not different among mated moths with complete versus incomplete ligations at all time periods (Table 2).

Injection of male reproductive gland extracts. Normally mated (positive control) females oviposited sooner and with more complete formation of the egg mass than any of the treated groups (Fig. 1). Virgins injected with one male tissue equivalent oviposited at approximately the same rate as virgins injected with the same volume of saline only. Untreated virgins (negative control) began egg deposition by day 4 as expected. The ovipositional response of moths receiving injections fell between the positive and the negative controls but most closely resembled the saline control.

Transplantation of spermathecae from ovipositing females into nonovipositing females. Transplant recipient virgins in all groups oviposited at approximately the same rate as sham transplanted control virgins, which oviposited later than normally mated females (Fig. 2). Both mated and transplanted females initiated oviposition sooner than untreated virgins, but this effect was due to the surgery as evidenced by egg output from sham transplanted females.

Effect of abdomen isolation and decapitation on oviposition. None of the isolated abdomens (n=3 for each time period) laid eggs during the three days following resection although they appeared to be alive. In the 5 and 7 h groups, 2 of 3 moths had begun oviposition prior to resection of the abdomen but did not continue laying eggs after isolation. To further evaluate this phenomenon, moths were decapitated 1 h (n=2) and 3 h (n=3) after initiation of copulation so that the thorax and

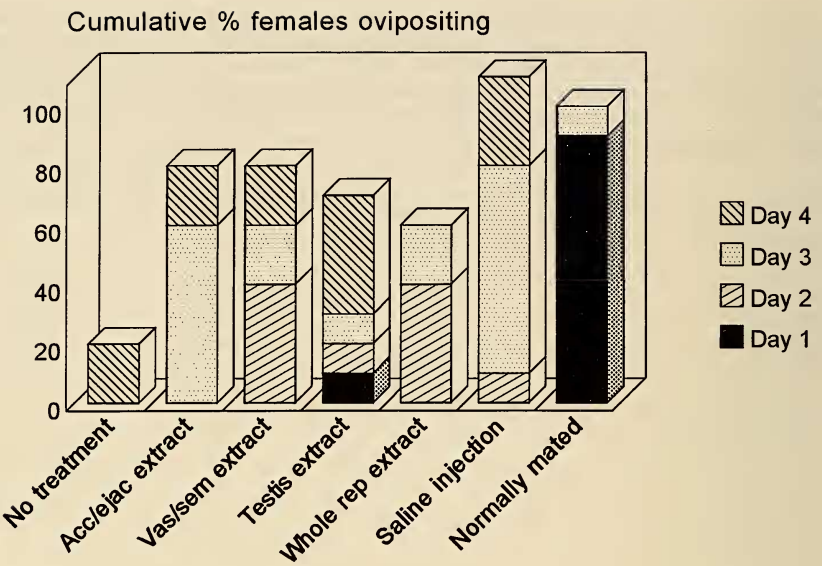

FIG. 1. Gypsy moth oviposition through 4 days following injection with extract of male reproductive tissue. Extracts injected into virgin females were prepared from: (a) accessory gland/ejaculatory duct; (b) vas deferens/seminal vesicle; (c) testis; and (d) whole male reproductive tract. Negative controls included untreated and saline injected virgins; the positive control consisted of normally mated females. N=10 for all treatments.

abdomen were intact. Although 1 moth in the 3 h group began laying eggs prior to treatment, none of the moths oviposited following decapitation.

Effect of bursa stimulation. Females were stimulated artificially by introducing a probe and injecting saline into the bursa. Glass rods (0.2 mm diam., 10 mm long) were inserted into the bursa and either gently manipulated for 2 min and removed or left in place for the duration of the experiment. Oviposition by moths at 5 days posttreatment was no greater than that by virgins handled the same way but without rod insertion. In another experiment, injection of 60–70 ml of Ringer's saline into the bursa through fine-tipped, polyethylene tubing also did not induce oviposition greater than that observed in negative controls.

DISCUSSION

If oviposition in gypsy moths were induced by a male-derived humoral factor acting on the brain, then early ligation should prevent transmission of the factor, causing a delay in egg laying. Similarly, late ligation should have no effect and treated females should behave as if they were normally mated. In these studies, ligated females oviposited

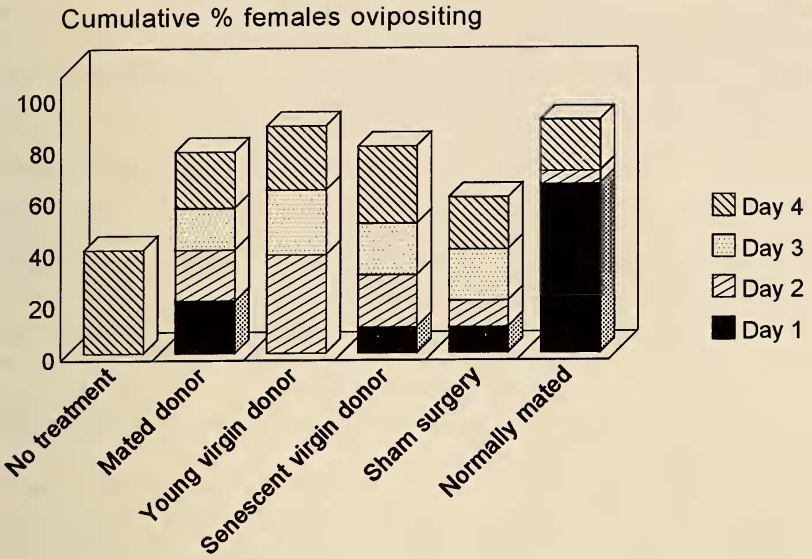

FIG. 2. Gypsy moth oviposition through 4 days following transplantation with spermathecae from various donors. Virgin gypsy moths received a spermatatheca from one of 3 donor types: (a) 1-day-old mater, ovipositing female; (b) senescent, ovipositing virgin; and (c) 1-day-old non-ovipositing virgin. Negative controls included untreated and sham transplanted virgins; the positive control consisted of normally mated females. N=8–20 per treatment.

at the same time as nonligated females and both groups produced comparable egg masses regardless of when a thoracic-abdominal ligature was applied (Table 1).

At best, the data in Fig. 1 suggest that females injected with semen- and sperm-containing extracts (the three middle groups in the figure) began oviposition a day or two earlier than those receiving accessory gland extract or saline. Injections of gypsy moth male reproductive gland extracts, including the highly secretory upper vas deferens (Riemann & Giebultowicz 1991), did not stimulate oviposition in virgin females comparable to the mated rate. Studies of male paragonial gland extracts have shown these methods to be successful for demonstrating humorally active, male-produced oviposition factors in Diptera (Leahy & Craig 1965, Riemann & Thorson 1969, Morrison et al. 1982, Chen et al. 1988). Moreover, in *Bombyx mori* (L.) (Bombycidae) injection of a saline or water extract of whole reproductive tract stimulated oviposition in virgin females (Yamaoka & Hirao 1977) whereas extract of abdomen (minus reproductive tract, alimentary canal and malpighian tubules) produced only a minor effect. In addition, whole reproductive extract

added to *in vitro* preparations containing the terminal abdominal ganglion increased the spontaneous firing of associated motoneurons. Yamaoka and Hirao (1977) provided evidence in Lepidoptera for a humorally transported, oviposition-stimulating component in male reproductive tract that also appears to exert a neural effect.

Our experiments involving transplantation of spermathecae from actively ovipositing females into fecund virgins elicited the same ovipositional response as transplants from nonovipositing virgins. Giebultowicz et al. (1990b) likewise reported no effect when transplanting spermathecae from females 1.5 to 3 h after mating into virgins.

The current tagmatal isolation studies suggest that oviposition is not solely controlled by the abdominal or thoracic ganglia. The head appears to be important in initiating oviposition since mated, decapitated moths did not oviposit but intact, ligated moths did. Perhaps the command to begin egg laying originates in the brain.

Several investigators have postulated that in Lepidoptera the events resulting in decreased pheromone production also may initiate oviposition (Thibout 1979, Sasaki et al. 1983, Sasaki & Riddiford 1984, Stringer et al. 1985, Giebultowicz et al. 1990a). Giebultowicz et al. (1991) showed that a 2 min stimulation of the bursa either by male genitalia or by a thin glass rod caused a temporary decline in gypsy moth pheromone production; the spermathecae needed to be present for up to 5 h after mating to result in permanent suppression of pheromone production and calling behavior. In our studies, mechanical stimulation of the bursa failed to elicit premature oviposition by virgins.

Giebultowicz et al. (1990b, 1991) suggested that stretch receptor nerves in the spermathecae of *L. dispar* mediate an ovipositional response after the spermathecae have filled with sperm. In their studies of females mated to males kept in constant light as pharate adults, a spermatophore was formed in the bursa but few or no sperm migrated to the spermathecae and females failed to lay eggs. This is similar to the work of Klatt in 1920 (referenced in Benz 1969) who showed that castrated *L. dispar* males, which produced spermatophores without sperm, did not stimulate females to oviposit. Both of these studies concur with a 1952 study by Behrenz (referenced in Giebultowicz et al. 1990b) who showed that oviposition was reduced in *L. dispar* females in which the spermathecae had been removed or ligated to prevent sperm migration.

Given these earlier findings and our lack of strong evidence for humoral mediation involving the brain, we agree with Giebultowicz et al. (1990b, 1991) that sperm interacting with the spermatheca is the predominant inducer of oviposition in this laboratory strain of the gypsy moth. However, it remains unclear whether the sperm act humorally or neurally. Giebultowicz et al. (1990b, 1991) suggested that stretch recep-

tors surrounding the spermatheca send a signal to initiate oviposition, either through a neural or neurohumoral sequence. An equally plausible explanation is that sperm receptors lining the spermathecae release a paracrine factor that acts independently of or in concert with nervous stimuli. Neither of these hypotheses explains the onset of oviposition by senescent virgins.

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